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Autoradiographic localization of mouse brain adenosine receptors with an antagonist ($[^3\text{H}]$ xanthine amine congener) ligand probe

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Abstract

A $[^3\text{H}]$ xanthine amine congener (XAC), a potent adenosine receptor antagonist, binds in a saturable and reversible fashion to high affinity binding sites in mouse brain ($B_{\text{max}} = 323 \pm 17$ fmol/mg protein, $K_d = 1.4 \pm 0.4$ nM). Adenosine receptor agonists and antagonists are more potent than adenosine uptake inhibitors in displacing the binding of $[^3\text{H}]$ xanthine amine congener ($[^3\text{H}]$ XAC). The anatomical distribution of $[^3\text{H}]$ XAC binding sites is consistent with its being a ligand probe for adenosine receptors. High binding site densities were observed in the hippocampus (stratum oriens and radiatum, molecular layer), superior colliculus (superficial gray), cerebellum (molecular layer), cerebral cortex and substantia nigra. The availability of a high affinity antagonist radioligand probe like $[^3\text{H}]$ XAC for adenosine receptors allows the comparative quantitative autoradiographic analysis of agonist and antagonist binding to adenosine receptors, e.g. under varying in vitro incubation conditions (presence and absence of guanine nucleotides and cations).

Keywords

Xanthine amine congener; Cyclohexyladenosine; Adenosine receptor; Autoradiography; Brain; Mouse

Radioligand probes have been valuable tools for the investigation of adenosine receptors in the mammalian brain [2, 13, 15]. The use of agonist radioligand probes such as $[^3\text{H}]$ cyclohexyladenosine ($[^3\text{H}]$ CHA) and 5'- N - $[^3\text{H}]$ ethylcarboxamide adenosine demonstrated differential localization of adenosine A₁ and A₂ receptors in autoradiographic studies [6, 9, 10]. Differential binding characteristics of adenosine receptor agonists and antagonists have been demonstrated in brain membrane [5, 11]. While antagonist binding was highly invariable, the affinity of agonist ligands was increased in the presence of cations

and decreased in the presence of guanine nucleotides. Interpretation of autoradiographic studies using an agonist ligand probe is therefore complex. However, the lack of high affinity antagonist ligands for adenosine receptors prohibited the use of an antagonist radioligand probe in autoradiographic studies. A functionalized congener approach to adenosine receptor antagonists has recently provided several candidates for high affinity antagonist radioligand probes [7] and the binding characteristics to cerebral cortical membranes of one of them, [^3H]xanthine amine congener ([^3H]XAC), a derivative of 1,3-dipropyl-8-phenylxanthine, has been described [8]. We now report the kinetic and pharmacological characteristics of [^3H]XAC binding, its regional distribution and the effect of 5'-guanylimidodiphosphate (Gpp(NH)p), a non-hydrolysable guanine nucleotide and MgCl_2 on [^3H]XAC binding in mouse brain slices. Results are compared with those obtained with [^3H]CHA in analogous experiments.

Young adult male mice (30 g) were sacrificed by decapitation, their brain rapidly removed and fast-frozen by immersion into -50°C isopentane, then stored at -70°C until used. Twenty micrometer thick sections were cut in a Bright-Hacker cryostat at -14°C , thaw-mounted on gelatin-coated microscope-glass slides, airdried, then stored at -70°C until used. Incubation conditions for [^3H]XAC binding in membrane preparations [8] were slightly modified. The sections were preincubated for 30 min in 50 mM Tris-HCl pH 7.4 buffer containing 1.5 IU/ml adenosine deaminase (Sigma, St. Louis, MO) at room temperature. Incubation was then performed for 2 h in 50 mM Tris-HCl pH 7.4 buffer containing varying concentrations of [^3H]XAC (157 Ci/mmol; DuPont NEN, Wilmington, DE) at 0°C . In experiments investigating the effect of 100 μM Gpp(NH)p (Sigma) and 2 mM MgCl_2 on [^3H]XAC binding, the incubation was performed at room temperature. Non-specific binding was defined in the presence of 20 μM CHA (Calbiochem, La Jolla, CA). At the end of the incubation period, sections were rinsed twice for 4 min in ice-cold Tris-HCl buffer followed by a brief immersion in H_2O . The experimental conditions for [^3H]CHA (27 Ci/mmol; DuPont, NEN, Wilmington, DE) binding were essentially as described before [1]. In saturation and displacement experiments sections were wiped off the slides with Whatman GF/B filters and the radioactivity measured by liquid scintillation spectrophotometry. For the autoradiographic experiments, the sections were dried under a stream of cold, filtered air and placed in apposition to LKB-ultrafilm in Wolf-Picker cassettes for 4 weeks. The films were developed in Kodak D-19 (4 min) and fixed in Kodak rapid fixer (3 min). Quantification was performed as described before [4] using Amersham [^3H]microscales.

Scatchard analysis of saturation experiments to mouse forebrain sections demonstrates high affinity binding of [^3H]XAC and [^3H]CHA with both ligands labeling approximately the same number of sites (Fig. 1). Adenosine receptor agonists and antagonists were both more potent than adenosine uptake blockers in inhibiting [^3H]XAC binding. Inhibition constants (K_i) were as follows: for the agonist CHA, 28 ± 14 nM; for the antagonist 1,3-dipropyl-8-*p*-sulfophenylxanthine, 229 ± 101 nM; and for the uptake blockers 6-(2-hydroxy-5-nitrobenzyl)thioinosine, 8.1 ± 1.1 μM and dipyrindamole, 23.7 ± 9.3 μM . The values are means $\pm$ S.E.M. of 4 experiments with 5 different concentrations of each inhibitor and a concentration of [^3H]XAC of 1 nM.

The autoradiographs show the regional distribution of [^3H]XAC and [^3H]CHA binding sites (Fig. 2). Highest binding site densities for both ligands were seen in various hippocampal layers as revealed by quantitative analysis in 4 different brains. In adjacent sections of the same 4 brains, and under otherwise identical experimental conditions, inclusion of 100 μM Gpp(NH)p into the incubation medium decreased [^3H]CHA binding to between 20% (molecular layer of cerebellum) and 60% (caudate-putamen) of control values, while [^3H]XAC binding was not markedly affected. The addition of 2 mM MgCl_2 to the incubation increased [^3H]CHA binding as expected to between 150% (layers of parietal cortex) and 300% (CA1 layers of hippocampus) of control values. Surprisingly, [^3H]XAC binding was decreased under these conditions to between 15% (layers of parietal cortex) and 40% (caudate-putamen) of control values.

We have demonstrated that the binding of [^3H]XAC to mouse brain slices shows similar characteristics as [^3H]XAC binding to rat brain membranes: K_d about 1 nM, nanomolar inhibition constants (K_i) of adenosine receptor agonists and antagonists, no effect of Gpp(NH)p [8]. The inhibition constant of CHA (28 nM) probably represents a mixture of a high and a low affinity constant typical for the inhibition of [^3H]XAC binding by an agonist [8] which were not separated under our experimental conditions (5 data points). Since [^3H]XAC is an antagonist and [^3H]CHA an agonist, the guanine nucleotide Gpp(NH)p and the cation salt MgCl_2 have different effects on their binding. The observed effects of Gpp(NH)p and MgCl_2 on [^3H]CHA binding and of Gpp(NH)p on [^3H]CHA binding are in good agreement with many previous reports [3, 5, 8, 11]. The decrease in binding of [^3H]XAC in the presence of MgCl_2 was unexpected. However, magnesium has been stated to decrease the affinity of 1,3-diethyl-8- $^{[3\text{H}]}$ phenylxanthine for adenosine receptors in EDTA-washed rat brain membranes [12], an observation we could confirm for [^3H]XAC in mouse brain membranes (unpublished observations). A similar effect in sections for [^3H]XAC would lead to a reduction in labeling as described.

In cerebral cortical membranes [^3H]XAC preferably labels A_1 -adenosine receptors [8]. In addition, [^3H]XAC has been used as a radioligand for A_2 -adenosine receptors in human platelet membranes [14]. [^3H]XAC like [^3H]CHA intensively labels A_1 -receptor areas like the CA1 region of hippocampus and the molecular layer of cerebellum. Further studies analogous to those performed by Lee and Reddington [9] under different experimental conditions (non-specific binding not defined in the presence of the A_1 -selective adenosine receptor ligand CHA, but e.g. XAC itself) have to be performed to evaluate to which extent [^3H]XAC labels brain A_2 -adenosine receptors.

In conclusion, the availability of [^3H]XAC as a high affinity ligand probe for adenosine receptors will allow the analysis of antagonist binding properties in future autoradiographic studies. As binding of [^3H]XAC may vary with cation concentrations, interpretation of changes in binding will be similarly complex as in the case of an agonist radioligand probe.

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Fig. 1.

Scatchard analysis of representative saturation experiments of [3 H]XAC and [3 H]CHA binding to mouse brain sections. Mouse forebrain sections were incubated with [3 H]XAC in concentrations from 0.05 to 10 nM and [3 H]CHA in concentrations from 0.4 to 40 nM. Non-specific binding was defined in the presence of 20 μ M CHA. Binding parameters obtained were: [3 H]XAC, $B_{\max} = 323$ fmol/mg protein, $K_d = 1.4 \pm 0.4$ nM; [3 H]CHA, $B_{\max} = 397 \pm 53$ fmol/mg protein, $K_d = 1.6 \pm 0.2$ nM (mean $\pm$ S.E.M. of 4 experiments with 8 concentrations of 3 H-ligand).

Fig. 2.

Photomicrographs of autoradiograms depicting the distribution of [^3H]XAC and [^3H]CHA binding sites in sagittal mouse brain sections. Adjacent sections were incubated with either 0.5 nM [^3H]XAC or 2 nM [^3H]CHA. Non-specific binding defined in the presence of 20 μM CHA was between 35% (0°C) and 40% (room temperature) for [^3H]XAC and 10% for [^3H]CHA under these conditions. For 9 gray matter and two white matter structures, optical density values were obtained in 4 different brains, transformed into fmol/mg dry tissue values using Amersham [^3H]microscale standards and the mean calculated ($n = 4$, S.E.M. lower than 25% in gray matter areas). Highest binding site densities (for [^3H]XAC, 75–100 fmol/mg dry tissue and for [^3H]CHA, 100–150 fmol/mg dry tissue) were seen in hippocampus (stratum oriens (O), stratum radiatum (R) and molecular layer (M)) and the superficial gray (SG) of the superior colliculus. High binding (for [^3H]XAC 50–75 fmol/mg dry tissue and for [^3H]CHA, 50–100 fmol/mg dry tissue) was also observed in layers of parietal cortex (CC), the molecular layer (ML) of cerebellum and in the substantia nigra (SN). Lower binding site density (for [^3H]XAC, 25–50 fmol/mg dry tissue and for [^3H]CHA 25–50 fmol/mg dry tissue) was measured in the caudate-putamen (CP) and the granular layer of cerebellum. Even lower binding site densities are present in white matter areas like corpus callosum and cerebellar white matter.